Analysis Genetic Stability in *Prunus humilis* Bunge Plants after Cryopreservation Twice

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Abstract

Cryopreservation is one of the important biotechnologies. The Shoot-tips of *Prunus humilis* Bunge were successfully cryopreserved using vitrification method. Eight single-bud sibling lines were established as model system for genetic analysis. Amplified fragmentation length polymorphism (AFLP) assay was used to detect the variation of genetic stability. In the detected sites, only two sites where band pattern changed after cryopreservation, might be contribute to the change in DNA methylation status at *Eco*R Ⅰ recognition according to the different sequence following the recognition. About one third bands which had changed after cryopreservation twice seemed to have cumulative effect. Methylation sensitive amplified polymorphism (MSAP) assay was carried out for further investigation DNA methylation changes. A significant change in DNA methylation status induced by cryopreservation was detected. Variety of changes of band patterns could be classified to four kinds.

Keywords

Cryopreservation; Cumulative Effect; MSAP; DNA Methylation; Prunus Humilis Bunge

Abbreviations

AFLP = Amplified Fragment Length Polymorphism; MSAP = Methylation Sensitive Amplified Polymorphism

Introduction

Cryopreservation store for plant materials in liquid nitrogen as a long-term preservation method of plant germplasm has many superiors over classical methods such as the avoidance of subculture during storage. So far, cryopreservation has been successfully applied to preserve many plants (Hirai et al, 1998, Harding, 2004), and then many kinds of cryopreservation technology have been developed. Cryopreservation was applied in preserving not only conventional plant germplasm but also medicinal- and alkaloid-producing cell lines, hairy root cultures, genetically transformed and transformation-competent culture lines. In recent years, a new application was developed for obtaining virus-free plants through cryopreservation the shoot-tips of plant (Wang and Valkonen, 2007). As an important technology, the genetic stability in recovered plants is one important aspect of concern. As with all in vitro conservation techniques, cryopreservation and the subsequent regeneration steps have the potential to induce somaclonal variation, which can reveal itself as alterations in morphology, chromosome number, gene expression, protein profiles, or DNA sequences (Harding,2004). Some reports have suggested that the DNA sequence wouldn't change after cryopreservation for Heredity homogeneity material (Scocchi et al, 2004; Hao and Deng, 2001; Sisunandar et al, 2010). In contrast, De-Verno et al. found variant RAPD fragments in two clones of white spruce (Picea glauca) after cryostorage and subculture (De Verno et al, 1999). It should be recognized that cryopreservation process essentially is one kind of the stresses, cryopreserved materials may different from materials non-cryopreserved such as promoting chimerism disintegration (Fukai et al, 1994). It is essential to determine whether the cryopreserved materials are genetically identical to the materials without cryopreservation. Epigenetic information is the same important as genetic information of DNA sequence,

which is also the essential part of plant inheritance diversity. Some researches showed that DNA methylation has been implicated in gene regulation (Ronemus et al, 1996; Ramchadani et al, 1999), transgene silencing (Bender, 2004) and genomic imprinting (Lund. et al, 1995). DNA methylation change could be caused by various biotic and abiotic stress factors thus producing epigenetic mutation (Kovarik et al, 1997; Labra et al, 2002). So it is necessary to investigate the changes of epigenetics in the preserved materials. Epigenetic aspect, such as DNA methylation change, is another concern on the cryopreservation, and some results showed that change in DNA methylation status accompanied enhancement of the capacity to root during the period of cryopreservation (Hao and Deng, 2001). A study employed a technique termed amplified methylation polymorphism (AMP) to detect genome-wide epigenetic methylation changes after cryopreservation of papara showing varying levels of methylation modifications (0.52–6.62%) of detected markers (Kaity et al, 2008). However, there is no report on the cryopreservation of plant twice and the genetic stability so far.

Prunus humilis Bunge is one of the fruits originated from China and famous for its high content of calcium element. We have developed a procedure for cryopreservation the specie (Zhang et al, 2007). In order to test the effect of cryopreservation to the DNA methylation of plant and to investigate whether changes in DNA methylation had the cumulative effect, cryopreservation was carried out with the vitrification technique twice using eight single-bud sibling lines of *Prunus humilis* Bunge as materials, and the methylation-sensitive amplification polymorphism (MSAP) technique, a relatively new modification of amplified fragment length polymorphism (AFLP) (Reyna-Lopez et al,1997), was used to test the DNA methylation.

Materials and Methods

Plant Material

Eight single-bud sibling lines of *Prunus humilis* Bunge were established as a system for molecular examinations. To take eight shoot-tips from one tufted bud and culture them separately. Each of these shoot-tips propagated 5-8 buds after 30 days of culture, then these shoot-tips were subcultured. Each of them originated from a common tufted bud. The culture medium used in the above procedures was B5 medium containing 0.5 mg/l BA, 40 g/l sucrose and 7 g/l agar. All materials were maintained at 25℃ and on 12 h photoperiod (33 $\mu mol.m^{-2}.s^{-1}$). Materials used for following experiments were described as figure 1.

Procedures for Cryopreservation by Vitrification

Shoot-cuttings containing apical bud were cut from the tufted shoots. Shoot-cuttings were pre-cultured on the B5 medium supplemented with 0.5 $mg{\cdot}L^{-1}$ BA and 2 M Glycerol for 3 days. Then about 1.5-2.0 mm in length shoot-tips from pre-cultured shoot-tips were loaded in a solution containing 60% PVS2 (B5 medium containing 0.4 M sucrose, 30% glycerol 15% ethylene glycol and 15% DMSO) for 10-20 min at room temperature, and incubated in PVS2 for 60 min at 0℃ prior to a direct plunge into liquid nitrogen. After rapidly thawing in a water bath at 40℃, the shoot tips were washed fourth times with 1.2 $mol{\cdot}L^{-1}$ sucrose solution and transferred medium on containing 0.5 mg L^{-1}BA, 40 g L^{-1} sucrose and 7 g L^{-1}agar for 3d in dark prior to exposure to the light. Regenerated seedlings two-months old were cryopreserved using same procedure above.

Survival Rate

Survival rates were evaluated after 7 days of culture. Survival rate and expressed as percentage of the shoot-tips remaining green (Hao et al, 2002) were relative to the total number of shoot-tips used for cryopreservation.

DNA Extraction

DNA was extracted from about 1 to 2 g leaves using a CTAB method (Rogers and Bendich, 1985). The extracted DNA was used for molecular analysis.

Amplified Fragment Length Polymorphism (AFLP) Assay

Two restriction endonucleases, i.e., *Mse* Ⅰ and *Eco*R Ⅰ,

were used. *Mse* Ⅰ has a 4-bp recognition site, and *Eco*R Ⅰ has a 6-bp recognition site. In addition, sometimes *Eco*R Ⅰ is sensitive to methylation of cytosine at recognition site according to the different sequence following the recognition. DNA was digested with *Mse* Ⅰ and *Eco*R Ⅰ as described by Maheswaran et al (1997). Completely digested DNA was used for adapter ligation. *Mse* Ⅰ adapter sequence was designed as 5'-GACGATGAGTCCTGAG-3′ and 3′-TACTCAGGACTCAT-5′ while *Eco*R Ⅰ adapter as 5′-CTCGTAGACTGCGTACC-3′ and 3′-CTGACGCATGGTTAA-5′. After adapter ligation, pre-amplification was performed using *Mse* Ⅰ primer Mc 5′-GATGAGTCCTGAGTAA-3′ and *Eco*R Ⅰ primer Ea 5′-GACTGCGTACCAATTCA-3′. Subsequently, selective amplification was carried out using all 64 possible combinations of *Mse* Ⅰ primers Mcca, Mcta, Mcac, Mctc, Mcag, Mctg, Mctt, Mcaa and *Eco*R Ⅰ primers Eaac, Eaag, Eaca, Eact, Eacc, Eacg, Eagc, Eagg. The reactions for adapter ligation, pre-amplification and selective amplification are the same as those used in the original protocols. The products of selective amplification were denatured by adding 1/2 volume of fomamide-buffer (98% formamide, 10 mM EDTA, pH 8, 0.05% bromophenol (Sango) and 0.05% xylene cyanol) and heating for 4 min at 94℃, then moved into 0℃ immediately for 10 min. Electrophoresis was performed with aliquots of each sample on 6% polyacrylamide gel (acrylamide/bisacyylamide, 19:1) containing 7.5 M urea and 1×TBE for 2.5 h at 55 W. Then the gel was stained with silver.

Methylation Sensitive Amplified Polymorphism (MSAP) Assay

The MSAP was adapted from Cervera et al (Cervera et al,2002) Aliquots (250 ng) of were digested for 3 h at 37℃ with 3 U *Eco*R Ⅰ in 20 μl of 2 μl 10×H buffer (TaKaRa), after digestion, after digestion, DNA was precipitated and digested with 3 U *Hpa* Ⅱ in 20 μl of 2 μl 10×L buffer (TaKaRa) for 3 h at 37℃. In the second reaction, the same amount of *Prunus humilis* Bunge genomic DNA was digested for 3 h at 37℃ and then at 65℃ for 2.5 h with 3 U each of *Eco*R Ⅰ and *Msp* Ⅰ in 20μl of 2 μl 10×T buffer (TaKaRa). The DNA fragments from the two reactions were added separately to an equal volume of the adapter/ligation solution, and the ligation reaction was allowed to proceed at 25℃ for 2 h. The ligation mixture was the diluted 1:5 dilution with sterile distilled water, and used as the preamplification with *Eco*R Ⅰ + A and *Hpa* Ⅱ / *Msp* Ⅰ + T primers.

Preamplification was performed in a final volume of 10 μl with 10×buffer, 1.5 mM $MgCl_2$, 0.08 mM dNTP, 10 ng *Eco*R Ⅰ primer, 30 ng *Hpa* Ⅱ / *Msp* Ⅰ primers, 0.5 U Taq DNA polymerases and 2.5μl of the above diluted ligation product. Reaction conditions were 2 min at 94℃, 26 cycles of denaturation (94℃ for 30 s), annealing (56℃ for 1 min), extension (72℃ for 1min), and a final elongation step of 72℃ for 1 min.

For selective amplification, the pre-amplification product was diluted 1:20 with sterile distilled water. In this step, *Eco*R Ⅰ and *Hpa* Ⅱ / *Msp* Ⅰ primers with two additional selective nucleotides were used. All the sequence of the adapter and primer used has been list in Table1. The selective PCR was performed in a final volume of 10 μl following the protocol of Vos et al (1995). The reactions for denature, electrophoresis and stain the same as in AFLP assay.

Results

Survival

Seven days after culture, most cryopreserved shoot-tips of all single-bud sibling lines survived and remained green, while only a few died due to the injury associated with cryopreservation. As a result, all single-bud sibling lines survived cryopreservation with a high survival rate (Table 2). All shoot-tips grow into multiple shoot clumps directly (Fig 2). There was no obvious morphological variation with the seedlings.

AFLP Analysis

DNA samples of the non-cryopreservation, the first cryopreservation and the second cryopreservation materials from 8 single-bud sibling lines were digested by *Mse* Ⅰ and *Eco*R Ⅰ and amplified with 64 primer combinations. A total of 11093 scoreable bands was obtained with these primer pairs. It was found that two sites band pattern changed after cryopreservation

and 768 different bands appeared in the changed site. In those changed bands, 512 bands disappeared after first cryopreservation and 216 bands disappeared after second cryopreservation which didn't disappear after first cryopreservation (Fig.3). The difference in band number might be contributed to the cryopreservation-related DNA methylation at *Eco*RⅠ recognition site. To further investigate the DNA methylation change and assess the cumulative effect, the MSAP assay was performed since it is more powerful and specific than AFLP assay.

MSAP Analysis

Since *Hpa*Ⅱ is inactive when either of the two cytosines is fully methylated whereas *Msp*Ⅰ is sensitive only to methylation at the external cytosine, methylation of the internal cytosine would lead to the appearance of a fragment in the amplification product generated from the *Eco*RⅠ/*Msp*Ⅰ digest but not in that obtained from the *Eco*RⅠ/*Hpa*Ⅱ digest. Similarly, hemimethylation of either of the two cytosines would lead to the appearance of a fragment in the amplification product from the *Eco*RⅠ/*Hpa*Ⅱ digest but not the *Eco*RⅠ/*Msp*Ⅰ digest (Shao et al,2005).

Sixteen pairs of primers were used to detect cytosine methylation at the 5′-CCGG-3′ sequence of the *Prunus humilis* Bunge genomic DNA. Four types of MSAP bands were observed (Fig.4). These types of bands were classified according to the following scheme. TypeⅠ whereby MSAP bands were present in both *Eco*RⅠ/*Hpa*Ⅱ and *Eco*RⅠ/*Msp*Ⅰ lanes; typeⅡ whereby bands were present in *Eco*RⅠ/*Msp*Ⅰ lanes, but not in *Eco*RⅠ/*Hpa*Ⅱ lanes; typeⅢ whereby bands were present in *Eco*RⅠ/*Hpa*Ⅱ lanes, but not in *Eco*RⅠ/*Msp*Ⅰ lanes; And typeⅣ whereby bands were present in neither *Eco*RⅠ/*Hpa*Ⅱ lanes nor *Eco*RⅠ/*Msp*Ⅰ lanes in one single-bud sibling line but present in other single-bud sibling lines (Li et al,2002). Four kinds of changes of MSAP bands type were observed during cryopreservation. The first group represents bands pattern changing after the first cryopreservation also detected after the second cryopreservation. The second group represents bands pattern changing after the first cryopreservation but after the second cryopreservation they changed to original pattern. The third group represents bands pattern the same as original pattern after the first cryopreservation, and changing after the second cryopreservation. Others represent different from the first group, the second group and the third group.

A total of 3042 bands of type Ⅰ, Ⅱ, Ⅲ, and Ⅳ were generated from the three sources of leaves, 156 out of these bands pattern changed after cryopreservation (Table 3, Fig.5). After first cryopreservation, 14 *de novo* methylation sites were found while only 7 demethylation sites were detected contrast non-cryopreservation. After the second cryopreservation, 10 *de novo* methylation sites detected were equal to the number of demethylation site contrast bands after the first methylation. One third changes of bands pattern appeared no difference after first cryopreservation but they changed after second cryopreservation. However, no difference was observed in the total number of changes in bands pattern after the first cryopreservation from second cryopreservation: 96 bands pattern changed after the first cryopreservation while 95 after the second cryopreservation.

Discussion

Cryopreservation is the method of choice to preserve plant germplasm resources in the long term without subculture. At the temperature of liquid nitrogen, all cellular divisions and metabolic processes are stopped (Engelmann,2004). So it was hoped that the plant materials could thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, vitrification method prevents plant materials entirely from the crystallization-related damage without expensive apparatus. In this study, eight single-bud sibling lines of *Prunus humilis* were successfully cryopreserved using vitrification and high survival was obtained both after the first and second cryopreservation.

The AFLP analysis showed that the DNA fragments from non-cryopreserved, the first cryopreserved and the second cryopreserved samples were similar in number and size. Besides two different fragments were

detected in seven samples of non-cryopreserved, the first and second cryopreserved. The difference in band number might be contributed to the mutation of *Eco*RⅠ recognition site or the cryopreservation-related DNA methylation at *Eco*RⅠ recognition site according to the different sequence following the recognition. In these different fragments, 10 bands changed after the first cryopreservation while 4 bands changed after the second cryopreservation, implying that cumulative effect of DNA methylation likely occurred during two cryopreservation processes.

Subsequently, MSAP assay was performed for subtle detection on DNA methylation status. It was found that the number of DNA methylation changes after the first cryopreserved was equal to that of the following changes. Nevertheless, the sites of methylation and patterns of DNA methylation changes after the first cryopreservation were different from the second cryopreservation. This implied that the influence of the first cryopreservation on the stress of Prunus humilis Bunge genomic DNA methylation changes was the same as the second; however, the emphasis of stress has altered. Hao suggested that the regeneration rate of cryopreserved plant could increase due to demethylation(Hao et al,2002). In this study, it was found that the regeneration rate of Prunus humilis Bunge after cryopreservation has not increased. And the rate of demethylation more than de novo methylation after the first cryopreservation was in contrast with the rate of demethylation equal to de novo methylation after the second cryopreservation.

Interesting, about one third changed sites of methylation status seemed to have cumulative effects, and about another one third changed sites of methylation status can pass down to offspring via asexual reproduction. Those cumulative effects include de novo methylation, demethylation and other pattern of methylation. It seemed that different sites have different cumulative effects. The cumulative effects may be caused by the stress of cryopreservation. The stress of first cryopreservation changed methylation pattern of some sites. Those changes may lead to delicate change of chromatin, and may be advantageous to change the pattern of methylation during the second cryopreservation. However, the biological functions of those sites and cumulative effect were still unknown.

Conclusions

As one of important method for plant germplast conversation, cryopresevation has been used in many plants. However, the genetic stability should been concerned. Epigenetic information also is the component part of genetics. Our results showed that cryopreservation has not brought variation of DNA sequence detected by AFLP method, but brought DNA methylation variation in some loci. Close attention should be paid to the epigenetic variation brought by cryopreservation, especially the gene expression variation caused by epigenetic variation.

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TABLE 1 SEQUENCE OF ADAPTERS AND PRIMERS USED IN MSAP

	***Eco*R Ⅰ**	***Hpa*Ⅱ/*Msp*Ⅰ**
adapter1	5′-CTCGTAGACTGCGTACC-3′	5′-GATCATGAGTCCTGCT-3′
adapter2	3′-CTGACGCATGGTTAA-5′	3′-AGTACTCAGGACGAGC-5′
preamplification	5′-GACTGCGTACCAATTCA-3′	5′-ATCATGAGTCCTGCTCGG-3′
primer	(E-A)	(HM+T)
amplification	5′GACTGCGTACCAATTCAAC3′	5′ATCATGAGTCCTGCTCGGTCAA3′
primer	5′GACTGCGTACCAATTCAAG3′	5′ATCATGAGTCCTGCTCGGTCCA3′
	5′GACTGCGTACCAATTCACA3′	
	5′GACTGCGTACCAATTCACT3′	
	5′GACTGCGTACCAATTCACC3′	
	5′GACTGCGTACCAATTCACG3′	
	5′GACTGCGTACCAATTCAGC3′	
	5′GACTGCGTACCAATTCAGG3′	

TABLE 2 SURVIVAL OF *PRUNUS HUMILIS* BUNGE SHOOT-TIPS AFTER CRYOPRESERVATION

Serial number of single bud sibling lines	Bud number at first cryopreservation	The first survival rate (%)	Bud number at second cryopreservation	The second survival rate (%)
1	60	83.9±8.3	40	80.0±5.0
2	60	85.0±3.3	43	79.1±9.3
3	59	81.3±8.5	38	78.3±5.2
4	60	81.6±5.0	36	83.3±11.1
5	58	79.3±5.2	32	75.0±3.1
6	61	83.3±8.2	40	82.5±7.5
7	55	81.8±3.6	33	75.8±9.1
8	53	83.0±7.5	34	76.5±5.9

TABLE 3 MSAP BANDS PATTERN CHANGES AFTER TWICE CRYOPRESERVATION

	The first group	Number of the first group	The second group	Number of the second group	The third group	Number of the third group	others	Number of others
	I-II-II	3	I-II-I	9	I-I-III	6	II-IV-III	3
	I-IV-IV	2	II-I-II	3	II-II-IV	10	IV-II-III	1
	II-I-I	1	II-III-II	1	III-III-I	1	IV-I-II	1
	II-IV-IV	13	II-IV-II	1	IV-IV-II	15		
	III-I-I	1	III-IV-III	2	IV-IV-III	25		
	III-II-II	1	IV-II-IV	8				
	III-IV-IV	18	IV-III-IV	9				
	IV-I-I	1						
	IV-II-II	4						
	IV-III-III	17						
total		61		33		57		5

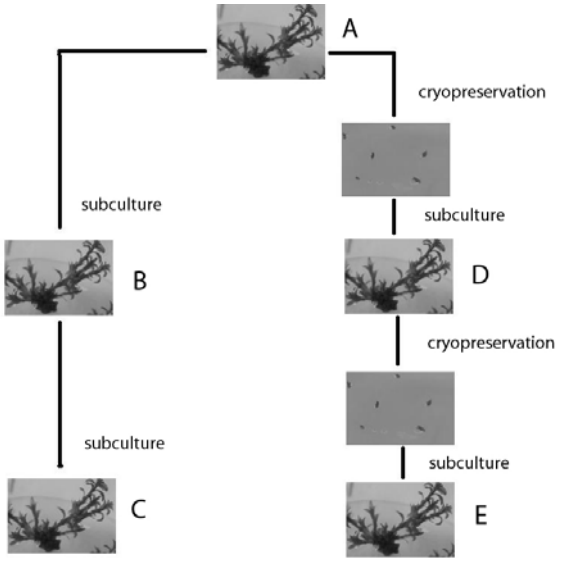

FIG. 1 AFLP patterns amplified from DNA template of the non-cryopreserved, the first cryopreserved and the second cryopreserved samples with primer pair M CTC-EAAG.
Lanes 1-1 to 8-1 are the AFLP patterns of 8 non-cryopreserved *Prunus humilis* Bunge single-bud lines, 1-2 to 8-2 are the those of 8 first cryopreserved samples and 1-3 to 8-3 are the those of second cryopreserved samples. The arrow points to the fragments that changed after cryopreservation.

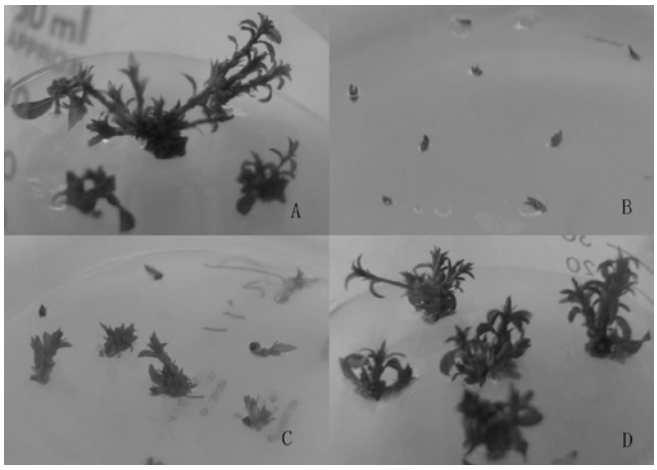

FIG. 2 MSAP patterns amplified from DNA template of the non-cryopreserved, the first cryopreserved, and the second cryopreserved samples of No.1 single-bud sibling line with primer pair EACC-HMTCAA.
The patterns of DNA methylation change were marked with ←, ⇦ which represent IV-II-II, IV-IV-I, respectively.

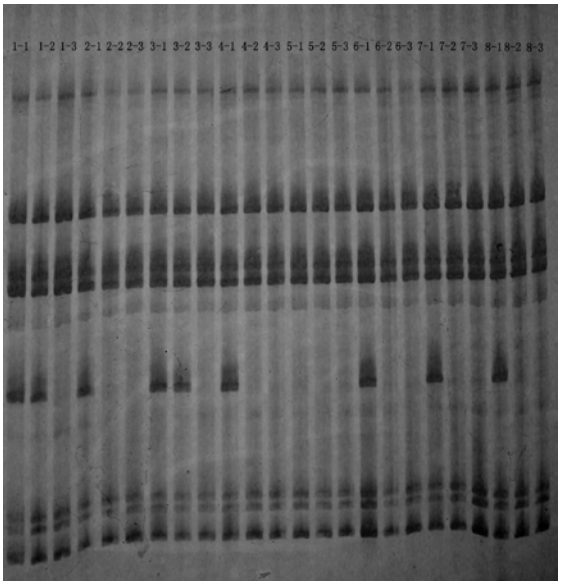

FIG. 3 AFLP patterns amplified from DNA template of the non-cryopreserved, first cryopreserved and second cryopreserved samples with primer pair M CTC-EAAG.

Lanes 1-1 to 8-1 are the AFLP patterns of 8 non-cryopreserved *Cerasus humilis* single-bud lines, 1-2 to 8-2 are the those of 8 first cryopreserved samples and 1-3 to 8-3 are the those of second cryopreserved samples. The arrow points to the fragments that changed after cryopreservation.

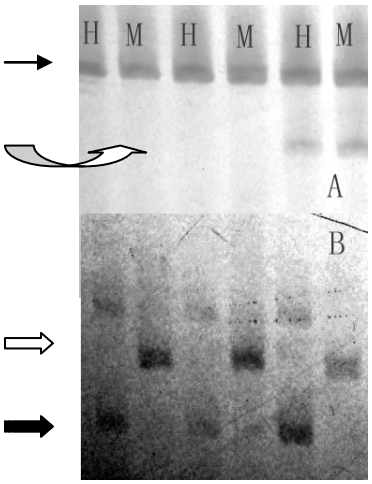

FIG. 4 Panel A and B refer to the two primer combinations EACC-HMTCAA and EACT-HMTCCA. Lanes H and M are the MSAP patterns specific to EcoRⅠ/HpaⅡ digest and EcoRⅠ/MspⅠ digest, respectively. Panel A and B represent genomic DNA extracted from leaves of No.1 single-bud sibling line. TypeⅠ bands were marked with →, while typeⅡ,Ⅲ,Ⅳ bands were marked with ⇨, ➡, ↷, respectively.

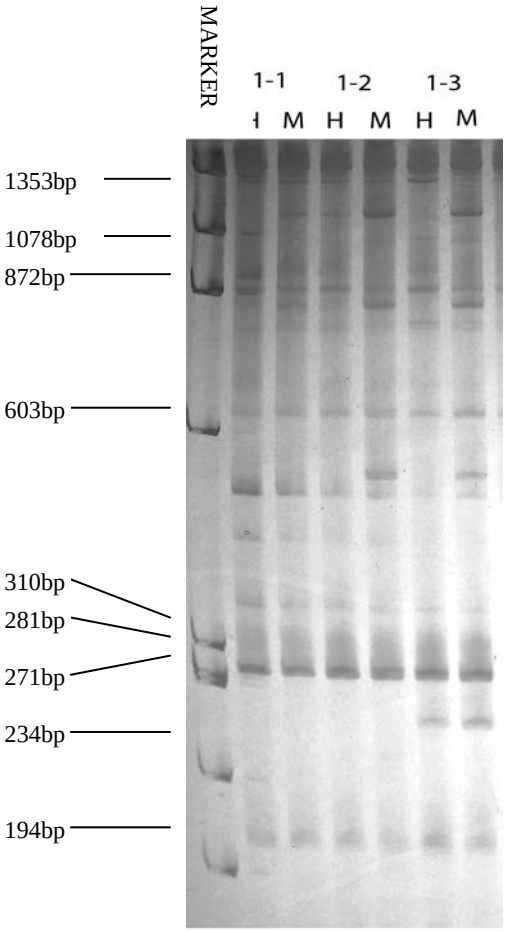

FIG. 5 MSAP patterns amplified from DNA template of the non-cryopreserved, the first cryopreserved, and the second cryopreserved samples of No.1 single-bud sibling line with primer pair EACC-HMTCAA.

The patterns of DNA methylation change were marked with ⇦, ⇦ which represent IV-II-II, IV-IV-I, respectiv